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(54) Title: PROCESS OF SEX SELECTION OF MAMMAL SPERMATOZOA AND METHOD TO CONTROL QUALITY OF FROZEN SEXED SEMEN DOSES

(57) Abstract: The invention allows the production by centrifugation in density gradient, in companies specialized in the production and commercialization of frozen semen, of semen doses enriched with X or Y chromosome-bearing spermatozoa, not impairing the fertilization capacity of said spermatozoa. According to the present invention, samples of spermatozoa containing both X and Y spermatozoa can be separated to produce subpopulations enriched with X or Y spermatozoa, which are substantially pure regarding the desired spermatozoa and substantially free from the other type of spermatozoa.

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Process of sex selection of mammal spermatozoa and method to control quality of frozen sexed semen doses

The invention allows the production by centrifugation in density gradient, in specialized companies for the production and commercialization of frozen semen, of semen doses enriched with X- or Y- chromosome bearing spermatozoa, without prejudice to the capacity of fertilization of said spermatozoa and which:

a) are compatible with the semen freezing and packing processes adopted by said companies;

b) may be commercialized for use in AI (Artificial Insemination) programs by using the traditional method (semen deposited shortly after the cervix) and under various reproductive handling conditions, obtaining pregnancy and birth rate of at least 75%;

c) may be used for IVP (in vitro production of embryos), obtaining cleavage and embryo development rates above 75% and 35%, respectively;

d) may be commercialized for use in conventional artificial insemination programs (births preferably of masculine or feminine gender) or those made after the hormone treatment of females to synchronize the heat and/or multiple ovulations and subsequent harvesting of embryos (most of them of feminine or masculine gender) and later transfer to recipient females.

According to the present invention, samples of spermatozoa containing both X- and Y- chromosome-bearing spermatozoa can be separated to produce subpopulations enriched with X- or Y-bearing spermatozoa, which are substantially pure regarding the desired spermatozoa and substantially free from the other type of spermatozoa. "Substantially free" means herein a sample of semen enriched with X spermatozoa which, when used in AI or IVP, has only a small chance to produce the birth of a male, since the spermatozoa sample has less than 20% and preferably less than 10% of Y spermatozoa.

Density gradients of the invention have a defined composition allowing to keep the viability of said spermatozoa, so that they become resistant against the conventional dilution and freezing processes and may, after thawing, keep acceptable viability levels (motility, power, % intact acrosome, etc.) for commercialization intended for conventional artificial insemination programs (births preferably of masculine or feminine gender) or those made after the hormone treatment of females to synchronize the heat and/or induce multiple ovulations and subsequent harvesting of embryos (most of them of feminine or masculine gender) and later transfer to recipient females. Furthermore, the production of semen may be intended for in vitro production of feminine or masculine embryos and later transfer to recipient females.

The method of the invention may be used to separate spermatozoa from various species of mammals, including humans.

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Different technological routes are taken in the attempt to select gender in mammals, both for zootechnical interest species and for species in extinction, pet animals and assisted human reproduction. There are two alternatives for that: to separate X-chromosome-bearing spermatozoa from those Y- chromosome-bearing spermatozoa; or to sex pre-implanted embryos.

To select the sex of bovine embryos, the potential market is restricted, since the volume of transfers corresponds to about 1% of the total of registered cows, both in Brazil and in countries such as the United States and Canada (data from the Brazilian Society for Embryo Transfer - SBTE).

In humans, embryo sex selection involves ethical issues that could be avoided by sexing spermatozoa. Spermatozoa sexing would have clinical use to prevent diseases linked to the X chromosome in the human species, such as e. g. Duchenne's muscle dystrophy, A or B Hemophilia, pigment retinitis leading to blindness, Lesh Nyan Disease or fragile X syndrome linked to mental retard (ZARUTSKIE et al, 1989). There are about 6000 heritable genetic abnormalities, of which more than 370 diseases are linked to the X chromosome (COTINOT et al, 1993; JOHNSON et al, 1993). Usually, diseases linked to X are expressed by children inheriting from the holder mother the X chromosome with anomalous genes. The possibility to select the sex to generate daughters may reduce or eliminate the probability to conceive affected sons.

The separation of X-bearing spermatozoa from those Y-bearing spermatozoa is based on the detection of at least one of the following phenotypic (chemical or physical) differences between these two types of cells:

a) pH sensitivity (EMMENS, 1960);

b) electrical load of the membrane surface (GORDON, 1957; KANEKO et al., 1984a);

c) morphology of the nucleus and head (SHETTLES, 1961);

d) surface antigens (GOLDBERG et al, 1971; KOO et al, 1973);

e) migration rate (ERICSSON et al, 1973; WANG et al, 1994);

f) DNA content (GARNER et al, 1983); and

g) density differences (WINDSOR et al, 1993).

BLECHER et al (1999) reported the identification and isolation of specific sex proteins (SSP, Sex Specific Protein) to obtain specific antibodies of masculine gender and feminine gender. After the incubation with bovine spermatozoa, specific anti-protein antibodies of feminine gender promoted agglutination of 50% of said cells. The cells that were not agglutinated produced 106 embryos in vitro, of which only 72 could be sexed by cytogenetics, of which 92% of the masculine gender. It was not reported if the incubation of spermatozoa with specific male anti-protein antibodies also developed the deviation in the sexual proportion in favor of the feminine gender. It is important to underline that the method was valid for being cytogenetic, and about 28% of

embryos could not be sexed under this technique. The authors claimed the presence of different sex-specific chromosomal proteins on the surface of X and Y spermatozoa, originating from post-meiotic transcription and translation which would be unable to cross the intersperm bridges. The authors consider that, after improving this new method, it would be possible to develop an immunological method to separate X and Y spermatozoa, although previous studies (HENDRDIKSEN et al, 1997, HOWES et al, 1997) had reported no success in identifying differences in membrane proteins between X and Y spermatozoa.

BLECHER registered in a patent (US 5,890,504; November 24, 1998) the identification of sex-specific molecules disclosed in his 1999 work, describing possible practical uses of his invention, reporting the development of a method for immunological sexage of spermatozoa by identifying sex-specific proteins (SSP). These methods are based on the identification and isolation of said proteins, their purification by column chromatography and obtaining anti-protein antibodies specific for the masculine gender and feminine gender. After the incubation with bovine spermatozoa, specific anti-protein antibodies of feminine gender induced agglutination of 50% of said cells. The cells that were not agglutinated produced 92% masculine gender embryos in vitro. It was not reported if the incubation of spermatozoa with specific male anti-protein antibodies also deviated sex ratio toward feminine gender. The authors claimed the presence of different sex-specific chromosomal proteins on the surface of X and Y spermatozoa, originating from post-meiotic transcription and translation which would be unable to cross the intersperm bridges.

An enhancement of this method is required for application in commercial scale and genetic improvement and animal production programs, since the authors have not reported the sex ratio deviation toward feminine gender, which is preferred in such programs. The author considers that preliminary results of molecule identification could in future be used in commercial scale for the immunological sexage of spermatozoa. Said possibility had already been disclosed by the literature and even patented by ZAVOS et al (US 4,999,283, March 12, 1991), entitled *Method for X and Y Spermatozoa Separation*, by using anti H-Y antibodies. Said method is based on the use of specific antibodies for the H-Y antigen - a specific transplant antigen for the masculine gender and well represented on the surface of spermatozoa (WACHTEL, 1983).

Studies to verify this possibility were conducted in mice by BENNET & BOYSE (1973) and in rabbits by ZAVOS (1983), who showed that the treatment with anti H-Y and complement, before artificial insemination, provided a small but significant deviation (8% toward females), considering that the sexual proportion is of 50% of feminine gender individuals. Subsequently, HOPPE & KOO (1984) did not obtain a deviation in sex ratio when they used, in vitro fertilization, mouse spermatozoa after this

treatment.

Despite the existence of various biological ways to prevent the haploid expression (OHNO, 1982) and the possible mRNA exchange due to the presence of cytoplasmatic bridges between X and Y spermatozoa (ERICKSSON et al, 1981), there is evidence in favor of the gene activity of germinative cells (BRADLEY, 1989). This author referred to various genes expressing in the haploid genome of germinative cells. He reports that murine spermatozoa, retained in an immunoaffinity column for H-Y antigen, produced 90% males when used in artificial insemination. In ovines, about 70-80% of spermatozoa that were not linked to the column have not presented fluorescence (H-Y negative) and about 80% of those who adhered to the column presented fluorescence (H-Y positive) after treatment with the anti H-Y antibody and conjugated antibody.

ALI (1990) sexed bovine spermatozoa, according to the expression of the H-Y antigen, treating them with anti-H-Y monoclonal antibody and conjugated antibody, submitting them to "fluorescence activated cell sorting". About 80% of fluorescent spermatozoa (H-Y positive) held the Y chromosome and 70% of those non-fluorescent (H-Y negative) held X.

PETER et al (1993) suggested that the separation of X- and Y-bearing spermatozoa by using monoclonal anti-H-Y antibodies linked to magnetized polymer beads (magnetic separation) may have about 98% precision. It is necessary, however, to repeat the methodology since results were obtained experimentally.

It is noteworthy that, being H-Y spermatogony positive, it is possible for X spermatozoa to retain H-Y antigen coming from those diploid cells on the membrane. This possibility was presented by some authors to explain the difficulties to sex rabbit and bovine spermatozoa by immunological methods (BENNET & BOYSE, 1973, OHNO & WACHTEL, 1978, KOO et al, 1979, ZAVOS, 1983, HOPPE & KOO, 1984, HENDRIKSEN et al, 1993).

Other authors describe methods to separate X- or Y-bearing spermatozoa by immunological techniques. SPAULDING (US 5,660,997, August 26, 1997) describes the use of proteins with sex-linked membranes (SAM) and suggests methods to change the sex ratio by using these proteins. However, the author does not effectively test any of the suggested methods. The confirmation that said proteins are effectively linked to spermatozoa and that said linkage has as a consequence the significant deviation of the sex ratio in any species of mammals is, therefore, lacking.

Finally, the most recently published patent by BENJAMIN et al (US 6,153,373, November 28, 2000) describes the use of non-porous magnetic beads linked to specific sex antibodies. Separation is made by adding to the semen antibodies against one of the genders, previously coupled to the beads. Spermatozoa remain for 30

minutes in contact with the antibodies in a homogenizer with 3 RPM rotation at room temperature. After this period, spermatozoa are submitted to a magnetic field by 15 minutes and the supernatant removed constitutes the fraction enriched with X or Y spermatozoa. For example, if anti-Y antibodies were used, the fraction enriched with X will be recovered in the supernatant and vice versa. Although the method described in that patent is widely known in immunology and therefore successful as for its efficiency, it had not yet been disclosed to separate these two spermatozoa populations. The author does not report the use of sexed spermatozoa in AI or any other approach that proved separation efficiency. Concerning the practical use of this method, the limitation persists as for the need of further investigations to prove the existence of a specific antibody for the masculine or feminine gender which could be efficiently employed.

Despite the existence of the Patents mentioned above about spermatozoa sexing by the immunological method, eletrophoretical studies of the membrane of X or Y spermatozoa separated by flow cytometry have shown that it is not possible to identify differences between membrane proteins from X- or Y-bearing spermatozoa (HENDRIKSEN et al, 1996; HENDRIKSEN et al, 1999).

The quantity of DNA between X and Y chromosomes varies significantly between species and it is so far the only scientifically established and valid difference to efficiently separate X or Y spermatozoa in vitro (JOHNSON, 1994). In bovines, this difference in DNA content reaches about 4.0% (GARDNER et al, 1983; JOHNSON, 1994). Based upon this difference, there are two methods that can be used to select the sex of spermatozoa: flow cytometry and density gradient centrifugation.

The difference between the quantity of DNA in X- or Y-bearing spermatozoa is evidenced by using a fluorescent coloring agent. After staining, X or Y spermatozoa are separated according to the intensity of fluorescence in a flow cytometer (JOHNSON & PINKEL, 1986; JOHNSON 1992, 1994). JOHNSON, in August 1992, in the U. S. Patent US 5,135,759, describes the cytometric method to separate these two spermatozoa populations. While still experimental, separation has been improved by the use of cytometers with high speed flow, such as MoFlo as discussed in various patents, US 5,150,313; US 5,606,039; US 5,602,349; US 5,643,796 and the international patent WO 96/12171.

The first publications on bovine spermatozoa sexing by using flow cytometry (US 5,135,759, Johnson) reported 90% precision when the flow speed is about 100 cells per second, which corresponds to about 2 x 10^6 spermatozoa each 5 to 6 hours. At that time, about 30 hours were required to separate the necessary quantity of spermatozoa to produce a dose of frozen semen for artificial insemination cervically, which is the conventionally used route (JOHNSON et al, 1994; SEIDEL et al, 1996). However, in the last few years, many studies were performed to optimize the results

obtained by this method. A few aspects were substantially modified to overcome technical limitations, which can be verified in the patents US 6,149,867; US 6,263,745; US 6,357,307 and WO 99/05504. For example, the variability of heterogeneity of the fluorescence signal of spermatozoa due to a small DNA variation (3 to 4%) in different species of mammals, on X or Y chromosomes, which is overcome by the use of a new funnel-oriented system. This new system, added to the use of cell separating equipment with high speed, promoted the separation of over 11 million X- bearing spermatozoa per hour, with 85 to 90% purity (JOHNSON & WELCH, 1999).

Thus we must stress the purity of spermatozoa separated by flow cytometry can be of around 85% when the separation speed of only one of the subpopulations is of 11 million/hour. However, when said flow speed is increased to 18 million/hour, the purity of spermatozoa falls to 75%.

Among the reasons that still limit the efficiency of this method, we can mainly underline the low quantity of viable sexed spermatozoa; the long exposure to toxic coloring agent under high temperature and the requirement to use semen in natura (JOHNSON et al, 1994).

With the increase of the efficiency of "in vitro" fertilization protocols, few spermatozoa are required to fertilize an oocyte. Therefore, the probability of the use of this method is higher. In bovines, by using 5 to 10 thousand sexed spermatozoa/oocyte, it is possible to separate, by flow cytometry, enough X spermatozoa per hour to fertilize up to 2200 oocytes. However, by using sexed spermatozoa, not submitting them to freezing, an only 66% cleavage index and 16 to 20% development index is reached (LU et al, 1999; GUTHIE et al, 2002). The use of frozen sexed spermatozoa may drastically reduce these rates.

The literature shows drastic reductions in fertility rates evaluated "in vitro" (penetration rate, cleavage rate, embryo development rate to blastocyst stage and number of blastocyst cells after spermatozoa sexing by cytometry in comparison to spermatozoa not submitted to sexing by this method (McNUTT and JOHNSON, 1996; RATH et al, 1997; BEYHAN et al, 1999).

JOHNSON (2000) underlines that the premature capacitation is clearly a characteristic of the sexed spermatozoa by the cytometer, which is a disadvantage for the spermatozoa intended to be frozen, but is an advantage for the spermatozoa to be immediately used after separation to make IVF, with no induction of the capacitation of spermatozoa before fertilization.

Another limitation is the reduced efficiency of thawing of sexed spermatozoa, since freezing prejudices the uniformity of nucleus staining, with coloring agent Hoechst 33342 (JOHNSON et al, 1994). This restricts the use of the best bulls (proven bulls) within each race in programs of animal improvement and progeny test

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using in vitro production of embryos.

Even though the treatments used in the flow cytometry method (staining with Hoechst 33342 and exposure to ultraviolet radiation) apparently do not inhibit the "in vitro" development of the zygote, LIBBUS et al (1987) noticed chromosomal aberrations in 50% of spermatozoa of *Microtus oregoni* after sexing by this method. DNA break frequency, for instance, was of 0.6 and 2.9 in the control group and in the one receiving treatment, respectively. The total number of chromosomal aberrations per cell reached 7.5.

Furthermore, in bovines, motility of sexed spermatozoa by this method was of only 51% for X or Y spermatozoa subpopulation (JOHNSON et al, 1997). After the freezing/thawing process, mortality decrease to 45% (laser regulated for 100 mW; 351, 364 nm) and only 65% of sexed spermatozoa maintained acrosome integrity (SCHENK et al, 1999).

These thawed spermatozoa (1 to 3 million/dose) when deposited in the body and horns of the uterus of heifers produced an average pregnancy rate of 48.5 and 50.5%, respectively. In this study, 180 pregnancies were obtained by using frozen X-bearing spermatozoa in AI in the body of uterus, of which 88 (81.5%) were female (ph) foetus, as diagnosed by ultrasound (SEIDEL et al, 1999). These factors, linked to the low number of separated cells/hour, make the commercial application of this method difficult and promote the investigation of another phenotypic difference between X and Y spermatozoa that can be discriminated for flow cytometry. An example is the work by VAN MUNSTER et al (1999a, b) confirming the existence of differences in head volume between X or Y spermatozoa and proposing them as an alternative parameter for sexing by flow cytometry. However, the authors have not yet disclosed the modifications to be required for the flow cytometry to make the sexing viable by using this parameter. Furthermore, there is no assurance that this new system keeps the sperm viability during the process, since other factors were not yet quantified and can prejudice said viability, such as:

a) the speed at which the flow of spermatozoa passes through the cytometer;
b) the temperature under which spermatozoa are maintained during the process; and
c) the time taken during the process to separate spermatozoa.

However, even if VAN MUNSTER et al are successful in developing a new cytometer, the high cost of acquisition (US$ 280,000) and maintenance of this equipment in comparison to the cost of acquisition (US$ 15,000) and maintenance of the refrigerated centrifuge (used in the present invention) makes spermatozoa sexing by centrifugation in density gradient becomes more promising for commercial use, since it produces higher quantities of sexed semen doses with higher quantity of live and viable spermatozoa at a lower cost than the flow cytometer.

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In equines, in which the usual dose of semen used in AI may reach 800 million to 1 billion spermatozoa, a drastic reduction of the insemination dose would be required to test sexed spermatozoa by cytometry. LINDSEY et al (2001) tested the use of low semen doses (5 million spermatozoa) in hysteroscopical insemination (uterine endoscopy) in four groups: non sexed and sexed, fresh and frozen/thawed. Pregnancy rate was the same for mares inseminated with fresh non sexed (40%), fresh sexed (37.5%) and frozen non sexed (37.5%) spermatozoa, but fell drastically with frozen sexed spermatozoa (13.3%). Unfortunately, the total number of pregnant mares in this experiment was only 18, which makes it difficult to draw reliable conclusions.

Despite the need to use semen doses with very low quantity of spermatozoa (compatible with cytometer production) and low sperm viability after sexing, flow cytometry has been the most studied method by various groups the last twenty years, mainly due to the sexing precision, which reached 95% (GARNER, 2000). However, literature review indicates that fertility results in the field by using sexed spermatozoa by this method show favorable rates under strictly controlled experimental conditions. Thawed spermatozoa (1 to 3 million/dose), when deposited on the body of the uterus of heifers produced an average pregnancy rate of 48.5% and 50.5% when deposited on uterus horns (SEIDEL et al, 1999). In this study, 108 pregnancies were obtained by using X-bearing frozen spermatozoa in AI in the body or horn of uterus, of which 88 (81.5%) were foetus of the feminine sex, as diagnosed by ultrasonography (SEIDEL et al, 1999).

It is therefore difficult to conclude what the results would be in countries like Brazil, where cattle/raising is characterized by heterogeneous handling conditions. In this sense, we consider that, for the Brazilian reality, it is more interesting to choose to develop a low cost methodology reaching sexing accuracy of about 75%, but allowing for, under varied handling conditions, satisfactory fertility rates.

Some authors consider flow cytometry as the most promising method, since its separation accuracy is about 90%. However, cytometry is highly costly and the fertilization capacity of spermatozoa is significantly reduced (CRAN et al, 1995).

We stress that the investment in the technique of this patent is significantly lower than that for the production of spermatozoa sexed by flow cytometry (cost of US$ 280,000), since the refrigerated centrifuge costs in average US$ 15,000 and produces about 30 times more sexed semen doses than the flow cytometer in the same period of time and with the same efficiency.

As an example, there is data in the literature (JOHNSON and WELCH, 1999) showing that, by flow cytometry, it is required one hour to produce 18 million sexed spermatozoa with 75% purity and with reduced viability after thawing most of the time. Using the method object of the present invention, at least about 550 million

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sexed spermatozoa can be produced in the same period with up to 81% purity, and sperm viability can be kept at comparable rates to non sexed semen.

In the present invention, it is possible to produce from 8 to 15 million spermatozoa from a subpopulation (X or Y) per gradient tube, in 20 to 30 minutes of centrifugation, depending on the used gradient. Therefore, the volume of frozen semen produced depends on the centrifuge capacity and its increase depends on the purchase of other centrifuges. Thus, contrary to what occurs with the flow cytometry method, in the present invention, the quantity of semen doses produced may be largely increased with lower investment (purchase of a centrifuge) and with no purity loss of the separated spermatozoa. Furthermore, semen doses enriched with X or Y spermatozoa produced by the invention may have increased precision (birth or IVP – "in vitro" production - of embryos of the desirable sex) if associated to embryo IVP methods and management techniques (AI time, nutrition, etc.) which may promote a deviation of the sex ratio.

Sedimentation or centrifugation procedures of spermatozoa are based on the density difference existing between X- or Y-bearing spermatozoa. SUMNER & ROBINSON (1976) analysed by microinterpherometry the head of spermatozoa and verified that X-bearing spermatozoa have more DNA and nuclear protein than Y spermatozoa and this difference is proportional to the mass difference between the two types of cells. The analysis of X or Y spermatozoa from various species by measuring chromatides (MORUZZI, 1979) or by flow cytometry (GARNER et al, 1983) showed that the difference in DNA content varies from 2.8% in human species to 12.5% in *Microtus oregoni* (PINKEL et al, 1982a; JOHNSON & CLARKE, 1990). In domestic animals, DNA differences between X or Y spermatozoa vary from 3.5% to 4.2%, and in bovines it is about 4.0% (GARNER et al, 1983; JOHNSON et al.,1987a; JOHNSON, 1992, 1994). In this species, significant average difference in the DNA content of X or Y spermatozoa between breeds was observed, but not between individuals of the same breed. Between X or Y spermatozoa of Jersey bulls, the highest difference (4.24%) was observed in comparison with Angus (4.05%), Hereford (4.03%), Holstein (3.98%) and Brahman (3.73%) breeds, which presents the lowest difference. The high quantity of water and lipids contained in the head of spermatozoa, since DNA corresponds to only 18% of the mass, makes the difference in DNA content to produce a small difference in weight and consequently in density. In 1982, Meistrich (mentioned by WINDSOR et al, 1993) calculated that the difference in bovine DNA content of X or Y spermatozoa results in a density difference of 7×10^{-4} g/cm^3, or 0.06% of the density over X spermatozoa. The author emphasized that this difference makes the separation of the two types of spermatozoa possible, as long as gradients with high density resolution are used.

Various approaches have been used in attempts to separate X or Y spermatozoa by centrifugation in density gradient. The first reports use Ficoll

sodium metrizoate (HEGDE et al, 1977; KANEKO et al, 1983) or Percoll (KANEKO et al, 1983, 1984; UPRETI et al, 1988; IWASAKI et al, 1988).

HEGDE et al (1977) separated human X or Y spermatozoa in a solution with 8% Ficoll and 33.8% sodium metrizoate. The final density of the solution was 1.08 g/ml. Equal volumes of this solution and semen were centrifuged at 100 X g for 20 minutes. Spermatozoa from the higher and lower portions were treated with Quinacrine to evidence F-body. The sediment before and after centrifugation was observed as containing 45.22 ± 6.14% and 62.9 ± 3.61% of Y spermatozoa, respectively, which was statistically significant.

In 1983, KANEKO et al compared the efficiency of Percoll and Ficoll to separate human X or Y spermatozoa by gradient centrifugation. Ficoll solution consisted of 5.7% (weight/volume) of Ficoll 400 and 9.0% (weight/volume) of sodium diatrizoate, pH 7.6 and density of 1.077 ± 0.001 g/ml. After centrifugation at 250 X g for ten minutes, a significant difference of 53.0 ± 1.5% and 40.6 ± 1.8% of Y spermatozoa was obtained in the upper and lower portions, respectively. Percoll was diluted to prepare solutions of about 40% to about 80% Percoll. After centrifugation at 250 X g for 20 minutes, the authors reached a significant difference of 73.1 ± 3.3% and 27.4 ± 3.4% of Y spermatozoa in the upper and lower portions, respectively. Results were verified by analysis of F-body.

In 1984, KANEKO et al and IIZUKA et al (1987) observed that, in the human species, when spermatozoa are put on a 76.7% - Percoll solution and ultracentrifuged at 30,000 X g for 20 minutes, the sedimentation speed of the X spermatozoa is faster than Y spermatozoa. Considering that the easiness with which cells pass through the gradient influences its sedimentation speed, a gradient with 12 portions was tested with different Percoll concentrations (25% to 80%, with a 5% variation in Percoll concentration between them), in an attempt to separate X spermatozoa. Among the examined conditions, centrifugation at 250 X g for 30 minutes had the best result. Spermatozoa were recovered in the sediment with accuracy of 23.3 + 6.3%; only 6.4% ± 1.8% were Y spermatozoa, as verified by analysis of F-body. This result showed that about 94% of the spermatozoa in the sediment were X-chromosome-bearing spermatozoa . More than 90% of spermatozoa maintained motility after treatment.

In bovines, IWASAKI et al (1988) analysed the sexual proportion of embryos produced in vitro with spermatozoa separated by Percoll gradient. The stock solution of Percoll (1 part of saline solution 10 times concentrated and 9 parts of Percoll) was diluted in saline solution to produce solutions containing 30% to 90% Percoll. The following discontinuous gradients of Percoll were formed, by depositing 1.0 ml of each one of these solutions in tubes:

a) 50 to 85% with 5% intervals (8 layers) at 15 °C;

b) 30 to 80% with 5% intervals (8 layers) at 15 °C;
c) 30 to 80% with 5% intervals (11 layers) at 20 °C;
d) 30 to 90% with 10% intervals (7 layers) at 20 °C.

It was centrifuged for 15 to 40 minutes. The centrifugation was stopped when about 5% of spermatozoa put over the gradient were recovered, which depended on the temperature. The embryos obtained were treated with vinblastine sulphate and prepared for chromosomal analysis. The statistical analysis showed no deviation in the sex ratio after treatment with Percoll.

Also in bovines, SCHWIDERSKI et al (1991) and BLOTTNER et al (1993) used centrifugation in Percoll gradient to separate bovine X-or Y-bearing spermatozoa. The gradient consisted of 10 layers of 0.6 ml of Percoll solutions with concentrations ranging from 22% to 48%. This method provided enrichment of more than 75% of X or Y spermatozoa in the lower and upper portions, respectively, as verified by in situ hybridization. After two centrifugations, spermatozoa from both subpopulations did not show any significant difference concerning morphology. The induction of acrosome reaction showed normal capacitation. Spermatozoa from both portions were used for the in vitro production of embryos which were sexed by PCR, which demonstrated that the use of spermatozoa from the upper and lower subpopulations resulted in 75% and 92% of male and female embryos, respectively.

The experiments reported above were not made with enough repetitions to guarantee the repetition capacity of the method. Furthermore, the evaluation of the separation by F-body analysis has low reliability. Viability evaluation of spermatozoa after the process only concentrates on the motility parameter, which is not enough to estimate their fertilization capacity. In these studies, neither the possibility of freezing the spermatozoa after the treatment, nor the production in a commercial system were evaluated.

The centrifugation methods in density gradient with Percoll, both for this purpose and to separate viable spermatozoa for procedures such as AI and IVP, although satisfactory as Percoll allows high resolution level, were abandoned. Percoll was forbidden for human use due to the occurrence of endomethritis in women inseminated with previously centrifuged semen in density gradient composed by colloidal means whose composition contains silica (MAKKAR et al, 1999). In the present invention, bovine females were inseminated with spermatozoa separated in Percoll gradient. As opposed to the human species, bovine females seem not to have their fertility (pregnancy rate of 80%) compromised after insemination with spermatozoa treated with Percoll.

Due to the statistically significant difference of the DNA content of X or Y spermatozoa among bovine breeds (GARNER et al, 1983), we can observe that, in some breeds, reproduction capacity of the results can be optimized by

associating a NycoPrep portion (iodinated means) to the Percoll gradient (ANDERSEN & BYSKOV, 1997) or by developing new denser gradients, composed of iodixanol (OptiPrep), allowing high density resolution level.

Recently, NORLING and PERTOFT described in patent (WO 02/052244-A2; July 4, 2002) gradients whose densities varied from 1.05 to 1.30 g/ml. As in the other methods described above, these gradients were applied in experimental conditions by using the colloidal medium ReadiGrad® (RG) whose formulation was described in the co-depending patent SE 00 04271-3. In comparison with WO 02/052244-A2 mentioned above, in which the RG medium is used, the present invention has the following differences:

a) In WO 02/052244-A2, the density gradients to separate X or Y spermatozoa were tested in experimental conditions (page 6, line 16). In the invention of the present patent, the density gradients were tested and separated X or Y spermatozoa in industrial/commercial conditions.

b) In WO 02/052244-A2 (page 7, line 12), the medium used to produce isotonic solutions is ReadyGrad®, while in the present invention we used Percoll and iodixanol.

c) In WO 02/052244-A2, X or Y spermatozoa are separated only after three washing centrifugations, which have the following purposes:

- first washing centrifugation: made with appropriate density gradient to separate slight particles from ejaculation (page 7, lines 19 to 24);
- second washing centrifugation: made after the transfer of the recovered material from the bottom of the tube of the first centrifugation to a second tube over which the second gradient is deposited, with appropriate density to separate immature spermatozoa, cells and possible protozoa (page 7, line 26-31);
- third washing centrifugation: made after the transfer of the recovered material from the bottom of the tube of the second centrifugation to a third tube over which the third gradient is deposited, with appropriate density to separate bacteriae, viruses and spermatozoon heads (page 8, line 4-10).

In the invention object of this patent, gradients composed of isotonic solutions made from substances comprising colloidal particles or gradients made with isotonic solutions from iodinated components were developed, allowing in a single centrifugation to separate impurities (slight particles, immature spermatozoa, cells, bacteriae, etc.) of viable spermatozoa and, among these, separate X from Y spermatozoa. The reduction in the number of centrifugations allows, not changing the precision for separation of spermatozoa X and Y, to reduce the injuries caused to spermatozoa by successive centrifugations and handlings and thus preserve their resistance to the freezing process.

d) In WO 02/052244-A2, the separation of X and Y spermatozoa only occurs in the fourth

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centrifugation, after three washing centrifugations to take out impurities. In the present invention, the withdrawal of impurities, and the separation of X or Y spermatozoa are achieved in one single centrifugation. Alternatively, one can make a second centrifugation just to increase the separation accuracy of X and Y spermatozoa achieved in the first centrifugation.

The introduction of iodinated components (developed for use as contrast in procedures using X rays) as a means for density gradients offered new opportunities to improve the methods to separate biological material, especially cells and subcellular organelles.

Iodixanol is a non-ionic iodinated component developed for studies involving X rays (NOSSEN et al, 1990; BOLSTAD et al, 1991) characterized for being a dimeric form of Nycodenz and having the double of its molecular weight. When separating subcellular organelles, iodixanol presents advantages over Percoll and Nycodenz, since it is dense enough to produce gradients with higher densities than 1.32 g/ml and lower osmolarity at 300 mOsm (FORD et al, 1994; GRAHAM et al, 1994).

In humans, centrifugation of semen in iodixanol gradient to separate viable spermatozoa is increasingly used by assisted reproduction clinics for both artificial insemination and in vitro production of embryos (HARRISON, 1997; SMITH et al, 1997; YANG et al, 1998; MAKKAR et al, 1999; McCANN et al, 2000). However, despite Iodixanol being an atoxic substance for therapeutical use in humans, its use in spermatozoa sexing protocols was not reported so far.

Commercial applicability of spermatozoa sexing depends on the establishment of a methodology which, is compatible with the freezing process, minimizes the loss of spermatozoa during the process and does not reduce their fertilization capacity. Considering these aspects, it is necessary to evaluate the quality not only concerning sexing accuracy, but also regarding the viability of the spermatozoa after thawing of frozen semen doses enriched with X or Y spermatozoa.

In the present invention, results after the insemination of cows with spermatozoa separated by centrifugation in the sexing gradients suggest that there was no interference of this process in fertility in vitro and in vivo.

So far, sexing spermatozoa protocols using centrifugation in iodixanol gradient were not found in the literature.

Patent documents regarding this issue will be analysed below on their applicability and functionality:

- Patents US 4,474,875 dated October 2, 1984 and US 4,327,177 dated April 27, 1982 (inventor: SHRIMPTON) separate X and Y spermatozoa based on their density difference, as well as the present invention, but use devices which could not be inserted among the equipments used in the semen freezing routine for commercialization and under

conditions not allowing to keep sperm viability, such as:

a) use of sedimentation for 24 hours in milk gradient;

b) use of temperature between -5 and +2 °C;

c) pH between 6 and 8; and

d) use of means with density between 1.01 and 1.15 g/cm^3 at 0 °C.

The design of the device used in the invention is composed of eight columns receiving 480 million spermatozoa each, of which only 21 million spermatozoa (4.5% of the total) are recovered from the bottom of each column. It should be stressed that, commercially, 21 million spermatozoa correspond in average to one semen dose. The purity of separation (about 75% of females) was verified in only 13 pregnancies. However, the author does not report the total number of inseminated females, i. e. how many attempts were made to obtain these pregnancies, which shows that a quality control process allowing the use in the artificial insemination industry was not developed or performed. On the other hand, the method of the patent was developed in such a way that sexed spermatozoa can be submitted to the control of sperm viability used in the routine of the companies producing frozen semen. Furthermore, a process to control the efficiency in separation of X or Y spermatozoa was developed, so that semen doses enriched with the X or Y subpopulations can be commercialized under the information of which should be the deviation in sex ratio after insemination. These are therefore completely different inventions, since the candidate method uses equipment and quality control test which can be inserted in the routine of semen freezing for commercialization and has the features to separate X or Y spermatozoa and keep sperm viability, since:

a) centrifugation is performed for only 10 to 20 minutes in density gradient composed of culture medium chemically defined to preserve the stability and integrity of spermatozoa membranes;

b) temperatures from 4 to 22 °C are used to preserve sperm viability; and

c) pH is stabilized at 6.8 to 7.4 associated to the presence of glucose, preventing early capacitation and acrosomal reaction.

- Patents WO 200151612-A1, WO 200195815-A1, US 5,135,759, US 6,149,867, US 6,357,307 and US 6,372,422 separate X and Y spermatozoa by flow cytometry. We clarify that flow cytometry and centrifugation in density gradient are fully different methods, since the latter:

a) makes use of methods preserving spermatozoon viability;

b) can be introduced in the routine of frozen semen production for commercialization. Item "b" is justified by the high cost of acquisition (US$ 280,000) and maintenance of the flow cytometer over the cost of acquisition (US$ 15,000) and maintenance of the refrigerated centrifuge.

- Patents US 5,439,362, US 5,660,997, US 5,840,504 and US 6,153,373 investigate the presence of different proteic markers on the membrane of X and Y spermatozoa by immunological methods to separate both subpopulations. However, eletrophoretical studies of the membrane of X and Y spermatozoa have shown that it is not possible to identify differences between membrane proteins of X or Y-bearing spermatozoa (HENDRIKSEN et al, 1996; HENDRIKSEN et al, 1999).

- Centrifugation methods in density gradients as presented in Patents US 5,314,074, US 5,840,502, US 6,390,966 are based on the density difference between two or more populations of cells or cell subunits, as well as other particles for separation. These are therefore widely used methods and not specifically developed for the sexing of spermatozoa.

The sale of previously sexed semen is such a highly expected event in the market that it has been announced for some years by the industries in the field, as we can verify in an article of the magazine DBO RURAL dated February 2000, in which GENUS-GENSEL previewed the start of commercialization of sexed semen within two years by ABS. The method as announced by GENUS is based on immunological methods (patent US 5,840,504) and forecasts that the sexed semen by this method will cost more than double than conventional semen, in the belief that, due to the cost, market demand (national and international) will be divided among both types, sexed and non sexed. In October 2001, GENUS-GENSEL ended its activities announcing that the technology they developed, despite competitive, is able to process small quantities of semen and still remains in the research stage. Another forecast for sale of sexed semen was made by XY Inc. (ARBL Building, CSU Foothills Campus, Fort Collins CO 80523, U. S. A.) in partnership with GEORGE SEIDEL (patent US 6,149,867) and was published in *Proceedings, The Range Beef Cow Symposium XVI*, December 1999, under the title: *Imminent Commercialization of Sexed Bovine Sperm*. In that article, the authors forecasted the commercialization in large scale of sexed semen by flow cytometry within two years. XY Inc. has licensed the method to the company COGENT, which introduced sexed semen in England commercially in July 2000. However, despite having 10 flow cytometry devices, MoFlo is able to produce a small number of doses (just to meet the local market). None of these forecasts came true, since the commercialization is restricted to the local market, with a small number of doses produced per year, much lower than worldwide demand. Furthermore, companies state that these doses will have low quantity of spermatozoa (in the tests, 1 to 3 x 10^6 spermatozoa per dose were used), which may make the use of this method to become unviable for the production of semen doses intended to conventional AI. The commercialization of sexed semen under this concentration will probably find barriers in the Brazilian legislation and in other countries which have the minimum quantity for commercialization of 10 x 10^6 live and viable

spermatozoa per dose.

The market demands, since the end of the 1920s, a technology for spermatozoa sexing that can be introduced in the production industry of frozen semen and that has the features contemplated by the present invention. Therefore, said invention does not change sperm viability to lower rates than those recommended by regulating agencies (e. g. Agriculture Department); it is compatible with freezing of spermatozoa after treatment for sexing; it allows the spermatozoa sexing after their thawing; and it allows the production of various doses of sexed semen (with precision of up to 86%) frozen per day at a compatible cost for the market.

The importance of this technology is its usefulness to maximize animal production at lower cost and it has been studied for many years. The possibility to produce, in commercial scale, semen doses enriched with X or Y spermatozoa will increase the benefits of the use of artificial insemination (AI), granting to it a decisive role to maximize the genetic progress between generations, according to the requirements of each genetic improvement program and livestock capacity.

Examples are the breeds specialized for milk production, in which the maintenance of pregnancies and birth of male animals is one of the factors reducing productivity and increasing production costs. Genetic progress will be maximized in breeding programs to produce milk, in which sex ratio is controlled at the time of artificial insemination to obtain males or females when desired (VAN VLECK et al, 1987; HOHENBOKEN, 1999).

In crossbreed programs, sex selection of spermatozoa will maximize productivity and allow to obtain higher proportion of males (75-90%) over females (10-25%; HOHENBOKEN, 1999). Due to the differences between sexes in the carcass characteristics (the carcass weight after slaughter of males at 24 months is about 25% higher than the carcass weight of females at the same age), the value of a progeny with 100% males will be about 34% higher than that composed of 40% males (RUVUNA et al, 1992). There are, however, breeding programs that can benefit from the birth of a higher proportion of females in the first generation (F1). These females are inseminated with semen from sires of a terminal breed (with carcass) characteristics and efficiency of food utilization allowing higher production of meat after slaughter (HOHENBOKEN, 1999). These programs are similar to those systems to produce meat with purebreds, in which maximum productivity is obtained when the full progeny is of females (TAYLOR, 1985).

In systems to produce meat using purebreds, efficiency of food utilization of the cows is inversely proportional to the number of calves composing its progeny. Therefore, in the meat production system, maximum efficiency is reached when heifers are mated, producing a female offspring to substitute it in the livestock, and is slaughtered shortly after its weaning ("single-sex bred heifer", or SSBH). The reasons are

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the following:

a) At that age, the cows assume the role of the product at slaughter, thus minimizing production costs of the calf; and

b) Higher number of parturitions decrease the efficiency of food utilization of the cow.

By controlling the sex by spermatozoa or embryo sexing, each cow can produce one or various heifers (by induction of multiple ovulations) in the first pregnancy and then be slaughtered after weaning said product (TAYLOR, 1985; HOHENBOKEN, 1999).

Furthermore, the use of sexed spermatozoa for in vitro production of embryos (IVP) will minimize the cost of progeny test, since it will only allow the birth of calves of the desired sex for zootechnical evaluation (e. g. females for the progeny test of bulls of milk breeds; NICHOLAS & SMITH, 1983).

Commercial applicability of spermatozoa sexing depends on the establishment of a methodology that is compatible with the freezing process, that minimizes the loss of spermatozoa during the process and that does not reduce their fertilization capacity. Considering these aspects, it is necessary to evaluate the quality not only concerning sexing accuracy, but also regarding the viability of the spermatozoa after thawing of the frozen semen doses enriched with X or Y spermatozoa. These aspects are critical to make the production of semen doses enriched with X or Y spermatozoa by the artificial insemination industry feasible at a compatible cost with market reality.

In bovines, AI is the main responsible for productivity increase. In Brazil, about 5% of females in reproduction age are inseminated (ASBIA 2000), with perspectives to reach 60 to 100%, such as in some developed countries as the USA and Japan, Australia and Denmark, respectively (BETTERIDGE, 1986). The increasing number of livestocks submitted to Genetic Improvement and Industrial Crossing Programs, which has had expressive increases since 1989, allowed the use of AI to increase 350% in the last ten years. These Programs keep semen sales above 5 million doses/year (ASBIA, 2000) currently. Besides zootechnical advantages (HOENBOKEN, 1999), this increase and potential market for AI justifies the huge interest in the spermatozoa sexing by semen collection or semen commercialization companies operating in Brazil.

Currently, the development of a method for spermatozoa sexing will also benefit assisted reproduction programs of animal species in extinction and pet animals (SEIDEL & JOHNSON, 1999).

Potential customers for the final product are in widely different markets, such as:

- AI companies;
- companies using in vitro fertilization (IVF) to make progeny test;

- IVF companies for use in commercial livestock; and
- reproduction clinics that provide genetic counseling and use IVF and AI.

The current market is bovine herds using AI and having compatible zootechnical control to the use of this technology. Another would be the commercial IVF programs or IVF programs for the progeny test.

Furthermore, a future market would be to adapt the method for human use, in case of genetic counseling and assisted reproduction, to minimize or eliminate the occurrence of diseases linked to the X chromosome.

Various methods described so far to separate X or Y spermatozoa involve complicated procedures with the use of sophisticated equipment that are still in development process to be adequate to the semen production industry, as the case of flow cytometer. Furthermore, all these methods, especially flow cytometry, cause irreparable damage to spermatozoa and their DNA, thus compromising the efficiency of the spermatozoa in the fertilization process and the production of embryos without DNA damage.

The separation performed by the present invention uses the method of centrifugation in density gradient, which is based on the density difference between X or Y chromosome-bearing spermatozoa.

The process to select sex of mammal spermatozoa object of the invention comprises the following steps:

a) centrifugation in density gradient composed of culture medium chemically defined to preserve the stability and integrity of spermatozoon membranes;

b) utilization of temperatures from 4 to 22 °C to preserve sperm viability;

c) pH stabilization within the physiological level for each species, preferably about 7.4;

d) association with the presence of substances preserving sperm integrity, preferably using glucose to prevent early capacitation and acrosomal reaction, especially in bovines; and

e) freezing of sexed spermatozoa in appropriate extender for this purpose, such as the use of Tris-egg yolk, for use in the in vitro production of embryos (IVP) or conventional artificial insemination (AI) (births preferably of masculine or feminine gender) or those made after the hormone treatment of females to synchronize the heat and/or multiple ovulations and subsequent recovery of embryos (most of them of masculine or feminine gender) and later transfer to recipient females.

The semen obtained from sires according to routine procedures can be used "in natura" or frozen.

More specifically, Percoll and Iodixanol (OptiPrep) gradients are used in the present invention. To obtain physiological conditions of pH and osmolarity, Percoll must be used in the form of a stock solution (SS) prepared by diluting 9 parts of

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Percoll (standard density of 1130 g/ml) in 1 part of culture medium DMEM (1:9, v/v); pH 7.4; 280-290 mOsm/kg H_2O. DMEM was prepared with ultrapure water, filtered in a membrane with 0.22 μ pores and stocked at 4 °C for periods not longer than 10 days. The pH was adjusted by using 1% glacial acetic acid or 1 N NaOH.

The OptiPrep solution is sold ready for use and there is no need to prepare stocks.

1. Animals

1.1 Donor semen sires

"In natura" semen was obtained from bovine sires that were in semen collection regime for freezing in a center of production of frozen bovine semen. Each sire had its first ejaculation collected with an artificial vagina as per routine procedures. 1 to 5 ml of the ejaculation were used for centrifugation in density gradient.

1.2 Artificial insemination

Crossbred females with ability to produce milk or meat were used for insemination. Two farms were chosen. These females were maintained in pasture and had access to mineral mix put in troughs distributed in fenced areas.

1.3. Preparation of Percoll and OptiPrep gradients

1.3.1. Preparation of Percoll gradients

1.3.1.1. Preparation of Percoll stock solution

The stock solution (SS) was prepared by diluting 9 to 11 parts of Percoll (standard density of 1130 g/ml) in 1 part of culture medium DMEM (1:9-11, v/v); pH 7.2 to 7.5; 280-320 mOsm/kg H_2O. DMEM was prepared with ultrapure water, filtered in a membrane with 0.22 μ pores and stocked at 4-6 °C for periods not longer than 7-10 days. pH was adjusted by using 1% glacial acetic acid or 1 N NaOH.

To obtain gradients, isotonic solutions (280-320 mOsm/kg) with different densities were prepared as follows:

Percoll Gradient: dilution, in different proportions, of SS (90% Percoll) in DMEM medium, containing 0.3 to 0.6% BSA; pH 7.2-7.5;

The discontinuous Percoll gradient was prepared by depositing 0.6 to 3 ml of each isotonic solution, from the denser one to the less dense, in conic tubes of polystyrene with the help of a pipette with adjustable volume.

The following gradients were tested:

Gradient 13 (G13): ten layers of isotonic Percoll solutions with densities varying between 1.034 and 1.068 g/ml;

Gradient 14 (G14): three layers of isotonic Percoll solutions with densities varying between 1.11 and 1.12 g/ml;

1.3.1.2 Preparation of OptiPrep gradients

The OptiPrep solution is sold ready for use and there is no

need to prepare stocks.

OptiPrep gradient: dilution, in different proportions, of OptiPrep in DMEM medium containing 0.3-0.6% of BSA, pH 7.2-7.5;

The discontinuous OptiPrep gradient was prepared by depositing 0,6 to 2 ml of each isotonic solution, from the denser one to the less dense, in conic tubes of polystyrene with the help of a pipette with adjustable volume.

The following gradients were tested:

Gradient 18 (G18): three isotonic layers of OptiPrep with densities varying between 1.123 and 1.163 g/ml;

Gradient 19 (G19): ten layers of isotonic Percoll solutions with densities varying between 1.034 and 1.070 g/ml;

1.4. Centrifugation and recovery of spermatozoa in the sexing gradients

60-120 x 10^6 viable spermatozoa/ml were deposited over each gradient.

Gradients 13 and 19 were centrifuged at 250-500 x g in a horizontal rotor for 8-20 minutes. After centrifugation, the portion rich in X spermatozoa, located at the bottom of the tube, and the one rich in Y spermatozoa, which is in the supernatant, were recovered. Both portions were separately submitted to recentrifugation in density gradient for 250-500 x g for 8-15 minutes at 4-8 °C.

In the recentrifugation of the portion rich in Y spermatozoa, the portion of supernatant was recovered and washed in DMEM medium with 250 to 500 x g centrifugation for 10 to 20 minutes at the same temperature of the gradient and re-suspended in a tris-egg yolk extender. The sediment resulting from the recentrifugation of that portion was discarded.

The sediment resulting from the recentrifugation of the fraction rich in X spermatozoa was recovered and, in Gradient 13, was washed in DMEM medium with 250 to 500 x g centrifugation for 10 to 20 minutes at the same temperature of the gradient and re-suspended in a Tris-egg yolk extender. The sediment of spermatozoa resulting from the recentrifugation of Gradient 19 was re-suspended in aTris-egg yolk extender. The supernatant resulting from the recentrifugation of that fraction was discarded.

The quantity of spermatozoa (concentration) resulting from the sexing process was verified and adjusted to 12 to 25 x 106/0.25 ml. Spermatozoa thus diluted were put in a cold chamber at 4 °C and packed according to company routine(s).

Gradients 14 and 18 were centrifuged at 250-500 x g in a horizontal rotor for 10-30 minutes at 18-25 °C.

The spermatozoa sediment resulting from the centrifugation in Gradient 14 was recovered, washed in DMEM medium with 250 to 500 x g

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centrifugation for 10 to 20 minutes at the same temperature of the gradient and re-suspended in a Tris-egg yolk extender. The sediment of spermatozoa resulting from the recentrifugation of Gradient 18 was re-suspended in a Tris-egg yolk extender.

The quantity of spermatozoa (concentration) resulting from the sexing process was verified and adjusted to 12 to 25 x 10^6/0.25 ml. Spermatozoa thus diluted were put in a cold chamber at 4 °C and packed according to company routine(s).

1.5. Spermatozoa freezing after centrifugation in density gradient

Spermatozoa recovered from the sexing gradient were frozen in a Tris-egg yolk extender according to the routine protocol of the company to be used for in vitro production of embryo (IVP) or artificial insemination (AI) using the commercial method.

1.6. Quality control of the semen doses containing centrifuged spermatozoa in density gradient

The quality control protocol for the fertility of spermatozoa submitted to sexing was composed by: a) evaluation of motility and power and Thermal Resistance Test (TRT); b) specific staining to evaluate the integrity of spermatozoa membranes; c) in vitro production of embryos; d) artificial insemination by the commercial method.

1.6.1. Evaluation of spermatozoa after thawing

A few doses of semen from each batch were thawed by the company for quality evaluation, with the following minimum values being admitted: motility (30%) and vigor (4) shortly after thawing (0 hour); and after the Thermal Resistance Test (TRT), motility (20%), vigor (3). Evaluations were made by the company technician who classified the semen batches as approved when they were above minimum standards and rejected when they were below. Only batches approved at TRT were used for AI.

1.6.2. Evaluation of the integrity of plasmatic membrane and acrosomal condition

Semen doses produced from different gradients and their respective controls were thawed in water bath at 35 °C for 30 seconds. Semen from each dose was deposited in a centrifuge tube. Three washes were made in a TALP culture medium added by BSA-V, centrifuged at 250 g for 10 minutes, aiming at extender removal.

After the last wash, the centrifuged sediment was suspended in 4.0 ml of TALP medium and two samples of semen of 10 microliter each were taken from each tube to proceed to the vital staining Tripan Blue/Giemsa.

One hundred spermatozoa were analysed per slide. Despite being possible to identify eight different classes of spermatozoa: 1) live intact (LI); 2) live damaged (LD); 3) live with detached acrosome (LS); 4) live with acrosomal reaction (LR); 5) dead intact (DI); 6) dead damaged (DD); 7) dead with detached acrosome (DS); 8)

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dead with acrosome degeneration (DR), in most of the analysed slides only spermatozoa of classes LI, LR and DR were found.

1.6.3. In vitro production of embryos

Semen doses submitted to centrifugation in density gradient and subsequently frozen were used for the in vitro production of embryos (IVP) using a methodology that eliminates factors that change the kinetics of blastocyst development and consequently sex ratio deviation towards masculine gender embryos.

Two doses of sexed semen, previously centrifuged in Percoll gradient for sex selection and frozen, were thawed at 35-37 °C for 20 to 30 seconds. The semen was deposited over a two-layers Percoll gradient (90 and 45%) at room temperature and centrifuged at 180 to 200 x g for 20 to 30 minutes, so to sediment viable spermatozoa. The sperm sediment resulting from this centrifugation had the volume measured and two samples of 5 microliter each were taken to determine progressive motility and concentration. Concentration was adjusted to 25 x 10^3 spermatozoa/ml with progressive motility.

Ovocytes obtained by follicle punction were maturated for 18 to 25 hours and washed three times in TCM-199 medium supplemented with 25 mM HEPES, 0.2 mM sodium pyruvate, 0.3% BSA fat acids free and once within IVF medium. Subsequently, they were put for fertilization (approximately twenty per 100 microliter drop and incubated for 15-30 hours in 5% CO_2 under the temperature of 38.5-39 °C).

After fertilization, probable zygotes had "cumulus" cells removed and were transferred to culture dishes, containing appropriate medium, added by 0.1% BSA serum-free. They were cultivated in atmosphere of 5% CO_2 in air, 100% relative humidity and temperature of 38.5-39 °C. Cleavage rate was evaluated after 18 to 30 hours of cultivation. Embryos from two to four cells continued to be cultivated under the same initial conditions up to the seventh day, when development was evaluated.

Selection and classification of embryos followed the rules established by the International Embryo Transfer Society (IETS), 1998.

1.6.4. Artificial insemination

With the purpose to evaluate fertility in field conditions, doses of frozen semen produced after the centrifugation of spermatozoa in the density gradients were used to inseminate heifers with ability to produce milk or meat. The observation of heat and artificial insemination were under the responsibility of the inseminators of the farm, who did not have any special training to perform these tasks on the cows intended for the sexing experiment. The observation of heat was made in the early morning and late afternoon. Females apparently accepting coupling were inseminated after 12 hours.

Pregnancy diagnosis and the sex of the foetus were verified

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by ultrasonography within 60 to 90 days after AI.

1.7. Validation of sexing results

1.7.1. Embryo sexing using PCR

The generic sex of embryos produced in vitro was identified by Polymerase Chain Reaction (PCR), using two pairs of different (oligonucleotide) primers amplifying two specific sequences of Y chromosome present in the genomic DNA of male bovines (XY). Primers were synthesized by PROMICRO (Sao Paulo - SP).

The two pairs of primers chosen for the specific sequence of Y chromosome were:

Primer 1 - 5' - CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT - 3'
5' - TGC TTG ACT GCA GGG ACC GAG AGG TTT GGG - 3'

Primer 2 - 5' - ATC AGT GCA GGG ACC GAG ATG - 3'
5' - AAG CAG CCG ATA AAC ACT CCT T - 3'

Primer 1 amplifies a 210 pb sequence for DNA of male bovines (BONDIOLI et al, 1989) and primer 2 amplifies a 250 pb sequence in males (SCHWERIN et al, 1991).

It was also used a specific primer for bovine DNA:

Primer 3 - 5'- AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA - 3'
5' - AAG ACC TCG AGA GAC CCT CTT CAA CAC GT - 3'

This primer amplifies a 280 pb sequence, repeated in the bovine genome (ELLIS & HARPOLD, 1986, 1988; ELLIS et al, 1988).

Each embryo was put in a tube with 0.2 ml capacity containing 10 microliter of ultrapure autoclaved water. The microtube was immersed in liquid nitrogen for 20-30 seconds and stored at -20 °C until the moment of PCR.

Before PCR, Proteinase K (16 mg/ml) was added to each tube containing an embryo and incubated for 60 minutes at 37 °C. Subsequently, Proteinase was denatured at 98 °C for 10 minutes. The contents of each tube were divided into two samples (A and B) which were subsequently submitted to PCR. We chose to divide DNA of each embryo into two samples in an attempt to assure the identification of the genetic sex in 100% of them and consequently make the validation of the sex ratio deviation by PCR reliable. In order to check the presence of contaminant DNA and verify the quality of reagents used in the reaction, each procedure had a negative control (with no DNA template), a masculine control (DNA of male bovine), a feminine control (DNA of female bovine),

Reaction conditions were as follows: 75 mM Tris-HCl, pH 9; buffer with 50 mM KCl, 2,0 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$; 1 U of Taq DNA Polimerase and 200 mM of dNTP. The total volume in the reaction mixture per tube was 30 microliter. In the tubes containing sample A, 10 pmol of primers 1 and 3 were added. In those

containing sample B, 10 pmol of primer 2 were added.

The amplification was performed in a Thermocycler PTC - 100 (M. J. Research, Inc. - Waltham, M. A. - U. S. A.). DNA of all samples was denatured at 94 °C for five minutes. Samples A were submitted to 40 x 94 °C cycles for 60 seconds, 58 °C for 30 seconds and 72 °C for 60 seconds, followed by an 8-minute period at 72 °C. Samples B were submitted to 38 x 95 °C cycles for 60 seconds, 58 °C for 60 seconds and 72 °C for 60 seconds, followed by a 5-minute period at 72 °C.

The PCR products were characterized by electrophoresis on 3% agarose gels, stained with ethidium bromide and visualized under ultraviolet light. Embryos were identified as females only when the 280 pb fragment was visualized in sample A and no other fragment was visualized in the corresponding sample B. The presence of two fragments (280 and 210 pb) in sample A indicated that the embryo was of masculine gender. This result was only confirmed when a 250 pb fragment was visualized in the corresponding sample B.

Gels were analysed by using photographic documentation equipment STRATAGENE (Eaglesight Software).

1.7.2. Foetal sexing by ultrasonography

Foetal sex was diagnosed by ultrasonography between 60 to 90 days after the artificial insemination, by using the Pie Medical Scanner 200 equipment, 5 MHz linear transducer.

1.7.3. Spermatozoa sexing validation by the quantitative real-time PCR method

There are two main ways to validate the separation and verify the fertility of spermatozoa after sexing:

a) IVP and later sexing of embryos; and

b) AI and subsequent Foetal sexing by ultrasound or observation at birth.

However, these validation methods, despite being scientifically faithful, are inappropriate in the routine of a company commercially producing sexed semen. To control the quality of each batch of produced sexed semen, the company should verify the accuracy of spermatozoa sexing, besides making routine tests.

Fertility parameters of the semen can be estimate in the laboratory and have a relatively high relationship with IA fertility rates. However, to verify the accuracy of X and Y spermatozoa separation, there is not yet a methodology accepted as faithful by the scientific community which can be easily performed, compatible with the routine of a company producing and commercializing semen. The available methods are the Barr corpuscle analysis (or F-body), whose reliability is questioned, or hybridization with male-specific probe, by using the FISH method (KING, 1984), which execution is troublesome, especially for a large number of samples. Furthermore, it is possible to verify the separation of X or Y spermatozoa using the flow cytometer, but this method is

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available in few laboratories worldwide and for a very high cost.

Spermatozoa sexing validation could be quicker by dispensing with embryo IVP protocol and utilization of another protocol to verify accuracy next to sperm separation.

DNA quantification procedures were reported in various studies as routine. The use of PCR to quantify X or Y chromosome-bearing cells of a sample, by using primers to amplify a fragment of amelogenin gene, was described by CHECA et al (2002). Ejaculates from bulls had the frequency of X and Y spermatozoa evaluated by using capillary electrophoresis of the amplification product.

Use of amelogenin gene for PCR sexing

During the formation of tooth enamel, ameloblasts secrete a proteic extracellular matrix formed by various groups of enamel proteins. Amelogenin constitutes the most abundant class of proteins, extremely important in the development of tooth enamel.

The amelogenin gene is located, only in bovines and humans, in the X and Y chromosome (LAU et al, 1989). In bovines, it is composed of six exons and five introns (GIBSON et al, 1991) and presents two different amelogenin transcriptions (class I and class II) which are products of genes located in the X and Y chromosome, respectively (GIBSON et al, 1991, 1992).

When a sequence of the amelogenin gene is amplified, it is possible to distinguish males from females, i. e. X and Y chromosomes, while using only a pair of primers, so that, during an amplification of said sequence, two very different fragments are observed, one from the amplification of the class I gene and the other from the class II gene. The amplification product from the class I gene (X chromosome) and class II (Y chromosome) is distinguished by a 67 pb deletion in the class II amelogenin gene (Gibson et al, 1991).

Description of the method to quantify X and Y chromosomes by PCR

DNA extraction from spermatozoa was made by the traditional method with phenol chloroform as per the protocol described by SAMBROOK et al (2001).

Semen was obtained from bulls in a collection regime in a commercial center and submitted to freezing according to routine of the company. Semen samples designated as sexed were previously centrifuged in density gradient with the purpose to select X spermatozoa. Samples of semen from the same ejaculation, but not submitted to X separation before freezing, were used as control.

For the PCR method, the protocol described by de ENNIS and GALLAGHER (1994) was used with a few modifications. In a final reaction mixture of 50 microliters, 24 ng of genomic DNA, 5 microliter of PCR buffer 10X, 1.5 mM $MgCl_2$, 0.2

mM each dNTP, 10 pmol of each primer, 1.25 units of Taq DNA Polimerase and ultrapure water were used. Samples were submitted to the following temperature cycle: an initial denaturation cycle at 97 °C for three minutes, followed by 25 denaturation cycles at 94 °C for one minute, annealing at 56 °C for one minute and extension at 72 °C for one minute. For final DNA extension, an additional cycle at 72 °C for ten minutes. Primers described by GIBSON et al (1991) were used:

5' - CAG CCA AAC CTC CCT CTG C- 3'

5'- CCC GCT TGG TCT TGT CTG TTG C – 3'.

Amplification products were analysed by the PCR real time equipment according to manufacturer recommendation. The frequency of X spermatozoa over Y spermatozoa is inferred by the number of copies of amplified fragments originated from the respective chromosomes:

a) fragment with 217 base pairs, originated from the amplification of class II amelogenin gene (located in Y chromosome); and

a) fragment with 280 base pairs, originated from the amplification of class I gene (located in X chromosome).

The present invention has the following advantages:

- it allows the production of semen doses enriched with X or Y spermatozoa by centrifugation in density gradient, in commercial scale, by a process compatible with the semen packing and freezing process of the specialized companies for the production and commercialization of frozen semen;
- it does not compromise the fertilization capacity of spermatozoa;
- it can be sold for use in AI (Artificial Insemination) programs by using the traditional method (semen deposited shortly after the cervix) and in various reproductive management conditions, obtaining pregnancy and birth rate of at least 75%;
- it can be used with frozen semen after thawing, which allows the use of the best bulls (proven bulls) in each breed in programs of animal improvement and progeny test using in vitro production of embryos, as opposed to the cytometry method, in which the freezing/thawing of spermatozoa reduces the sexing efficiency as the freezing disrupts the uniform staining of nuclei with Hoechst 33342 ;
- it can be used for IVP ("in vitro" production of embryos), obtaining cleavage and embryo development rates above 75% and 35%, respectively;
- the method of the invention can be used in sexing of spermatozoa in various species of mammals, including humans;
- the investment in the method of this patent is significantly lower than that for the production of spermatozoa sexed by flow cytometry (cost of US$ 280,000), since the refrigerated centrifuge costs in average US$ 15,000 and produces about 30 times more sexed semen doses than the flow cytometer in the same period of time and with the same

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efficiency; and

- the present invention can produce 8 to 15 million spermatozoa from a subpopulation (X or Y) by gradient tube, with 20 to 30 minutes of centrifugation, depending on the used gradient. Therefore, the volume of frozen semen produced depends on the centrifuge capacity and its amplification depends on the purchase of other centrifuges. Thus, as opposed to what occurs with the flow cytometry method, in the proposed invention, the quantity of semen doses produced may be largely increased with lower investment (purchase of a centrifuge) and with no accuracy loss of the separated spermatozoa.

- Semen doses enriched with X or Y spermatozoa produced by the invention can have increased accuracy if associated with embryo IVP methods and management techniques (AI time, nutrition, etc.) which may promote sex ratio deviation.

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Claims

1. Process of sex selection of mammal spermatozoa, from the obtainment semen in natura donors of masculine gender according to routine procedures, **characterized** by the following stages:

a) centrifugation in density gradient composed of culture medium chemically defined to preserve the stability and integrity of spermatozoa membranes;

b) utilization of temperatures from 4 to 22 °C to preserve sperm viability; and

c) pH stabilization within the physiological level for each species, preferably about 7.4;

d) association to the presence of substances that preserve sperm integrity, preferably using glucose to prevent early capacitation and acrosomal reaction, especially in bovines; and

e) freezing of spermatozoa after centrifugation in density gradient in appropriate extender for this purpose, such as the use of Tris-egg yolk, for use in the in vitro production of embryos (IVP) or conventional artificial insemination (AI) (births preferably of masculine or feminine gender) or those made after the hormone treatment of females to synchronize the heat and/or multiple ovulations and subsequent recovery of embryos (most of them of feminine or masculine gender) and later transfer to recipient females.

2. Process of sex selection of mammal spermatozoa, from the obtainment semen in natura donors of masculine gender according to routine procedures, **characterized** by the following stages:

a) centrifugation in density gradient composed of culture medium chemically defined to preserve the stability and integrity of spermatozoa membranes;

b) use of temperatures between 6 and 22 °C to preserve sperm viability;

c) pH stabilization within the physiological level for each species, preferably about 7.4;

d) association to the presence of substances that preserve sperm integrity, preferably using glucose to prevent early capacitation and acrosomal reaction, especially in bovines; and

e) use of semen for in vitro production of embryos (IVP) or artificial insemination (AI).

3. Process, in accordance with claims 1 and 2, **characterized** by the fact that the density gradients are composed of isotonic solutions made from substances composed of colloidal particles or iodinated components, preferably Percoll and Iodixanol (OptiPrep), diluted in culture medium.

4. Process, in accordance with claims 1, 2 and 3, **characterized** by the use of a culture medium associated to buffer components allowing to keep pH within the physiologic range for the spermatozoa of the used species, preferably pH between 7.2 to 7.5; with 280-350 mOsm/kg H_2O, with said culture medium DMEM preferably associated to the presence of substances preserving sperm integrity, plus a protein source, e. g. plus 3-6% BSA (Bovine Seric Albumin), said gradients

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prepared by means of isotonic solutions (280-350 mOsm/kg) with different densities prepared by dilution in different proportions.

5. Process, in accordance with claim 1, **characterized** by the inclusion of centrifugation and recovery of spermatozoa previously placed on the sexing gradients, centrifuged at an appropriate centrifuge force for each gradient in a horizontal rotor, long enough for X and Y spermatozoa to separate.

6. Process, in accordance with claim 1 **characterized** by the fact that, after centrifugation, the fraction enriched with X spermatozoa, located at the bottom of the tube, and the one enriched with Y spermatozoa, which is located in the supernatant, are recovered and alternatively both fractions are separately submitted to re-centrifugation in density gradient.

7. Process, in accordance with claims 1, 4 and 6, **characterized** by the fact that, in the recentrifugation of the fraction enriched with Y spermatozoa, the fraction of supernatant is recovered and washed or not in a chemically defined culture medium at the same temperature of the gradient and re-suspended in a diluter used in the routine of freezing spermatozoa for commercialization.

8. Process, in accordance with claim 1, **characterized** by the freezing of spermatozoa after centrifugation in density gradient, being said spermatozoa recovered from the sexing gradient and used for in vitro production of embryos (IVP) or artificial insemination (AI) using the commercial method.

9. Sexed semen, in accordance with any of the previous claims, **characterized** by the fact that such semen contains subpopulations of spermatozoa enriched with X or Y-bearing spermatozoa and is submitted to the following tests:

- quality control of the semen doses containing centrifuged spermatozoa in density gradient, being said fertility quality control protocol of spermatozoa submitted to sexing comprised of: a) evaluation of motility and vigor and Thermal Resistance Test (TRT); b) specific staining to evaluate the integrity of spermatozoa membrane; c) in vitro production of embryos; d) artificial insemination by the commercial method.

10. Method for artificial insemination, **characterized** by the use of the sexed semen of claim 9.

11. Method for in vitro production of embryos, **characterized** by the use of sexed semen of claim 9.

12. Method to verify the sex ratio deviation of embryos produced in vitro with sexed semen of claim 11, in which said PCR method is **characterized** by the detection of bovine embryos by using specific primers for the Y chromosome, (5' - CCT CCC CTT GTT CAA ACG CCC GGA ATC ATT - 3' / 5' - TGC TTG ACT GCA GGG ACC GAG AGG TTT GGG - 3') and primers for an autosomic

repetitive sequence of the bovine species (5'- AGG TCG CGA GAT TGG TCG CTA GGT CAT GCA – 3 / 5' - AAG ACC TCG AGA GAC CCT CTT CAA CAC GT - 3') in the same reaction, as well as a second verification with another specific sequence for the Y chromosome (5' - ATC AGT GCA GGG ACC GAG ATG - 3' / 5' - AAG CAG CCG ATA AAC ACT CCT T - 3').

13. Quality control method for the separation of X or Y spermatozoa using DNA method, by amplifying the amelogenin gene in the PCR including REAL TIME PCR system of claim 9, in which said PCR method is **characterized** by the fact that its quantifies the frequency of X and Y spermatozoa of the semen by using a sequence of the amelogenin gene (5' - CAG CCA AAC CTC CCT CTG C- 3' / 5'- CCC GCT TGG TCT TGT CTG TTG C – 3').

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(54) Title: PROCESS OF SEX SELECTION OF MAMMAL SPERMATOZOA AND METHOD TO CONTROL QUALITY OF FROZEN SEXED SEMEN DOSES

(57) Abstract: The invention allows the production by centrifugation in density gradient, in companies specialized in the production and commercialization of frozen semen, of semen doses enriched with X or Y chromosome-bearing spermatozoa, not impairing the fertilization capacity of said spermatozoa. According to the present invention, samples of spermatozoa containing both X and Y spermatozoa can be separated to produce subpopulations enriched with X or Y spermatozoa, which are substantially pure regarding the desired spermatozoa and substantially free from the other type of spermatozoa.

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3894529 A (SHRIMPTON) 15 July 1975 (15.07.1975) *column 15, lines 50-55; claims.*	1-13
Y	WO 2002/052244 A2 (AMERSHAM BIOSCIENCES AB) 4 July 2002 (04.07.2002) *page 1, lines 27-29; page 3, lines 17-20; examples; claims 1, 2, 11.*	1-13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT
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